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1 **Roscovitine exacerbates *Mycobacterium abscessus* infection**  
2 **by reducing NADPH oxidase-dependent neutrophil trafficking**  
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4

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25 **Keywords:** Cystic fibrosis; Roscovitine; CFTR; Neutrophil; NADPH oxidase; *Mycobacterium abscessus*;  
26 Inflammation; Infection; Innate immunity; Zebrafish  
27  
28

29 **Abstract**

30 Persistent neutrophilic inflammation associated with chronic pulmonary infection causes progressive lung  
31 injury and eventually death in individuals with cystic fibrosis (CF), a genetic disease caused by bi-allelic  
32 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene.

33 We therefore examined whether Roscovitine, a cyclin-dependent kinase inhibitor that (in other conditions)  
34 reduces inflammation while promoting host defence, might provide a beneficial effect in the context of CF.

35 Herein, using CFTR-depleted zebrafish larvae as an innovative vertebrate model of CF immuno-  
36 pathophysiology, combined with murine and human approaches, we sought to determine the effects of  
37 Roscovitine on innate immune responses to tissue injury and pathogens in CF condition.

38 We show that Roscovitine exerts anti-inflammatory and pro-resolution effects in neutrophilic inflammation  
39 induced by infection or tail amputation in zebrafish. Roscovitine reduces overactive epithelial ROS-mediated  
40 neutrophil trafficking, by reducing DUOX2/NADPH-oxidase activity, and accelerates inflammation resolution  
41 by inducing neutrophil apoptosis and reverse migration. Importantly, while Roscovitine efficiently enhances  
42 intracellular bacterial killing of *Mycobacterium abscessus* in human CF macrophages *ex vivo*, we found that  
43 treatment with Roscovitine results in worse infection in mouse and zebrafish models. By interfering with  
44 DUOX2/NADPH oxidase-dependent ROS production, Roscovitine reduces the number of neutrophils at  
45 infection sites, and consequently compromises granuloma formation and maintenance, favouring  
46 extracellular multiplication of *M. abscessus* and more severe infection.

47 Our findings bring important new understanding of the immune-targeted action of Roscovitine and have  
48 significant therapeutic implications for safety targeting inflammation in CF.

49

50

## 51 Introduction

52 Cystic fibrosis (CF) is a fatal disorder resulting from mutations in the cystic fibrosis transmembrane  
53 conductance regulator (CFTR)<sup>1</sup>. The leading causes of premature death in CF individuals is progressive  
54 pulmonary injury and respiratory failure caused by mucus obstruction, infections and inflammation<sup>2</sup>.

55 In CF lungs, impaired CFTR results in airway surface liquid dehydration and collapse of mucociliary  
56 clearance, predisposing to recurrent infections with a subsequent hyper-inflammatory profile<sup>2</sup>. CF infections  
57 are typified by pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia*  
58 *cenocepacia* or the non-tuberculous mycobacteria *Mycobacterium abscessus* (Mabs)<sup>3</sup>. In addition, CFTR  
59 deficiency results in abnormal activation of macrophage and epithelial cell responses to pathogens<sup>4</sup>,  
60 releasing pro-inflammatory mediators, such as IL8 and reactive oxygen species (ROS). This favours the  
61 onset of an exuberant influx of neutrophils<sup>4-7</sup>, which nonetheless fails to control infections and worsens lung  
62 function<sup>8,9</sup>. Moreover, defects in CFTR impair the ability of neutrophils to undergo apoptosis<sup>10-12</sup> and reverse  
63 migration<sup>7</sup> leading to increased neutrophil activity and longevity and therefore contribute to sustained  
64 pulmonary inflammation<sup>7,12</sup>. Evidence suggests that inflammation may even precede infection in CF  
65 airways<sup>13-15</sup>. Elevated inflammatory markers in the bronchoalveolar lavage fluid of CF infants are found, even  
66 in the absence of detectable infection<sup>16</sup>. In particular, we have demonstrated that CFTR dysfunction directly  
67 alters the response of epithelial cells to “sterile” injury and leads to exuberant ROS production through the  
68 DUOX2/NADPH oxidase, driving an overactive neutrophil response in a CFTR-depleted zebrafish model<sup>7</sup>.

69 Reducing the deleterious impact of inflammation is therefore an important therapeutic goal in CF<sup>17</sup>.  
70 Conventional anti-inflammatory therapies in CF include the use of glucocorticoids or ibuprofen which are  
71 potentially effective but associated with significant long term side effects<sup>18</sup>. CFTR modulators have been  
72 shown to reduce inflammation, however their high cost and mutation/age restriction preclude widespread  
73 use. Antibiotic treatment alone is insufficient to prevent inflammatory lung damage and can induce  
74 antimicrobial resistance. Although inflammation is reduced with anti-inflammatory treatment<sup>19</sup>, chronic  
75 inflammation remains a consistent feature, indicating a continued need for novel approaches to prevent  
76 inflammation-mediated tissue destruction in CF.

77 One potential and interesting alternative is represented by Roscovitine, an inhibitor of cyclin-dependent  
78 kinases (CDK)<sup>20</sup>. In particular, this compound is capable of inducing neutrophil apoptosis<sup>21,22</sup>, accelerating  
79 the resolution of inflammation<sup>23-25</sup>. Importantly, Roscovitine has proven beneficial in enhancing apoptosis of  
80 neutrophils isolated from CF patients<sup>11</sup>. However, the pro-apoptotic activity of Roscovitine has never been  
81 evaluated in *in vivo* models of CF. Roscovitine also exerts anti-inflammatory actions on macrophages<sup>26,27</sup>,  
82 eosinophils<sup>28,29</sup> and lymphocytes<sup>30</sup>. Moreover, Roscovitine enhances bactericidal activity of CF alveolar  
83 macrophages<sup>31,32</sup>. However, Roscovitine has not been tested in CF infection models. Roscovitine is currently  
84 being evaluated in a phase 2 clinical trial in CF patients infected with *P. aeruginosa*, as a potential anti-  
85 pseudomonas therapy <https://clinicaltrials.gov/ct2/show/NCT02649751?term=roscovitine&rank=1>.

86 Here, we demonstrate that Roscovitine can restore normal levels of inflammation in a *in vivo* model of CF  
87 by *i*) reducing epithelial ROS production-driven neutrophil mobilisation and *ii*) enhancing neutrophil apoptosis  
88 and reverse migration. Importantly, beside macrophage-directed bactericidal effect of Roscovitine, we show  
89 that Roscovitine promotes an increased susceptibility to Mabs infection *in vivo* by inhibiting DUOX2/NADPH

90 oxidase-dependent neutrophil trafficking. This study represents a clear demonstration of the protective role  
91 of DUOX2-mediated ROS production against Mabs infection.

92

93

94

95 **Methods**

96

97 Bacterial strains, human cells, mouse and zebrafish lines and detailed methods associated with all  
98 procedures below are available in **Supplemental Methodology**.

99

100 **Zebrafish experiments**

101 Zebrafish experiments were conducted according to guidelines from the UK Home Office under AWERB and  
102 in compliance with the European Union guidelines for handling of laboratory animals.

103

104 **Mouse experiments**

105 Mouse procedures were authorised by Ethics Committee A783223 (APAFIS#11465-2016111417574906).

106

107 **Macrophage experiments**

108 Primary human macrophages were generated from peripheral blood samples from consented healthy and  
109 individuals with CF volunteers (approved by regional ethics approval REC12/WA/0148).

110

111 **Quantification and statistical analysis**

112 Statistical analysis was performed using Prism 7.0 (GraphPad Software) and detailed in each Figure legend.  
113 ns, not significant ( $p \geq 0.05$ ); \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

114

## 115 Results

### 116 117 Roscovitine rebalances early neutrophil infiltration by epithelial ROS-dependent mechanisms

118 We first proceeded to examine the potential benefits of Roscovitine in reducing neutrophilic inflammation  
119 by exploiting the zebrafish model of sterile inflammation<sup>7,33,34</sup>. In zebrafish larvae, tail fin amputation triggers  
120 neutrophil infiltration towards wound, accurately mimicking the kinetics and fates observed in human  
121 inflammatory responses<sup>33,35</sup>. In particular, zebrafish neutrophils have the same function as human  
122 neutrophils and respond in a similar manner to chemicals, including Roscovitine<sup>23</sup>.

123 In order to investigate the effect of Roscovitine on neutrophilic response, we exploited the  
124 *TgBAC(mpx:EGFP)i114* line harbouring green-fluorescent neutrophils<sup>33</sup>, in normal and CFTR-deficient  
125 contexts, using *cftr* morphants (*cftr* MO)<sup>6</sup> or the knockout *cftr*<sup>sh540</sup> mutant (*cftr* -/-)<sup>7</sup>. To first address whether  
126 Roscovitine influences early neutrophil infiltration, injured-WT and CF larvae were incubated with  
127 Roscovitine, or *i*) the NADPH-oxidase blocker Diphenyleneiodonium (DPI), known to inhibit early neutrophil  
128 mobilisation<sup>7,36</sup>, *ii*) the pro-resolution drug Tanshinone IIA (TIIA), which does not influence early neutrophil  
129 chemotaxis<sup>7,37</sup> and *iii*) DMSO. Roscovitine treatment, but not TIIA, was able to reduce neutrophil influx in WT  
130 and CF injured-fish, effectively rebalancing overactive neutrophil mobilisation in CF to that of WT levels  
131 (**Figures 1A-B**). Interestingly, comparative analysis showed similar wound-associated neutrophil number in  
132 both DPI- and Roscovitine-treated larvae. Epithelial release of H<sub>2</sub>O<sub>2</sub>, through the DUOX2/NADPH oxidase, is  
133 required for the early neutrophil response to injury<sup>7,38,39</sup>. We then investigated the potential anti-oxidative  
134 action of Roscovitine on the recruitment of early-arriving neutrophils, by measuring ROS production in  
135 injured CF fish. Compared to DMSO-treated animals, microscopy revealed that Roscovitine caused a  
136 substantial inhibition of epithelial ROS production, as judged by decreased CellROX fluorescence intensity at  
137 the wound (**Figures 1C-D**). This finding suggests that Roscovitine modulates the earliest phase of neutrophil  
138 mobilisation to injury in an epithelial oxidase-dependent manner.

139 Collectively, these results indicate that Roscovitine reduces CF-associated inflammation by reducing both  
140 epithelial oxidase activity and early neutrophil influx to injured tissue in CFTR-depleted zebrafish.

### 141 142 Roscovitine-driven neutrophil apoptosis and reverse migration accelerate inflammation resolution *in* 143 *vivo*

144 CF zebrafish exhibit persistent neutrophilic inflammation after injury<sup>7</sup>. We therefore investigated whether  
145 Roscovitine treatment could resolve such a response to initiate regenerative processes.

146 WT and CF *TgBAC(mpx:EGFP)i114* larvae were injured and, 4 hours later, exposed to Roscovitine or  
147 DMSO. Roscovitine reduced established post-wounding neutrophilic inflammation in WT<sup>23</sup> and CF contexts  
148 (**Figures 2A-B**). Pro-resolution events such as local neutrophil apoptosis and migration of neutrophils away  
149 from inflamed sites play a critical role to reduce inflammation and restore tissue homeostasis<sup>37,40,41</sup>. We first  
150 examined the extent of neutrophil apoptosis *in vivo* in CF zebrafish. Combined confocal imaging and  
151 quantification of TUNEL-positive neutrophils showed that CFTR-deficient larvae treated with Roscovitine  
152 exhibited enhanced neutrophil apoptosis at wound at 8 hours post-amputation (hpA), compared to their  
153 control counterparts (**Figures 2C-D**). Interestingly, Roscovitine induces neutrophil apoptosis more efficiently  
154 than TIIA (**Supp 1A**). We then investigated whether Roscovitine could also influence neutrophil retrograde

155 migration by examining and comparing the dynamics of neutrophil reverse migration in DMSO- and  
156 Roscovitine-treated larvae using *Tg(mpx:Gal4)sh267;Tg(UAS:Kaede)i222* larvae (**Figure 2E**)<sup>7,42,43</sup>.  
157 Remarkably, Roscovitine significantly enhanced neutrophil reverse migration in injured CF fish (**Figures 2F-**  
158 **G**). However, Roscovitine is a much less potent inducer of neutrophil reverse migration than TIIA (**Supp 1B**).

159 Efficient inflammation resolution plays a pivotal role preventing tissue damage, as well as initiating tissue  
160 healing and repair<sup>44-46</sup>. The pro-resolution property of Roscovitine, linked to increased neutrophil apoptosis  
161 and reverse migration, prompted us to analyse tissue repair potential in zebrafish treated with Roscovitine.  
162 Despite evidence of reduced damage to regenerated tissues, our results indicated that defective tissue  
163 repair was not reversed by Roscovitine exposure in CF animals (**Supp 2A-B**).

164 Overall, we show that Roscovitine promotes resolution of established neutrophilic inflammation and  
165 alleviates inflammatory damage in CFTR-depleted fish by enhancing both neutrophil apoptosis and reverse  
166 migration.

167

### 168 **Roscovitine exposure compromises epithelial ROS-dependent neutrophil mobilization during Mabs** 169 **infection**

170 As neutrophils represent the first line of defence against invading bacteria, including the multi-drug  
171 resistant pathogen Mabs<sup>47,48</sup>, we were next interested in determining the effect of Roscovitine on neutrophil  
172 responses during Mabs infection, using a zebrafish model of Mabs infection<sup>49,50</sup>. Chemoattraction of  
173 neutrophils was assessed by injecting Mabs expressing *tdTomato* into the somite of *TgBAC(mpx:EGFP)i114*  
174 larvae as previously described<sup>48</sup>. As shown in **Figures 3A-C**, Roscovitine exposure resulted in a significant  
175 reduction in neutrophil mobilisation towards Mabs-infected tissue. Neutrophil chemotaxis is known to require  
176 functional epithelial ROS signalling<sup>51</sup>, suggesting this could also account for the Mabs-induced neutrophil  
177 response. While injection of Mabs consistently triggers oxidative responses in infected tissues, confocal  
178 microscopy showed abnormal oxidative activity in Roscovitine-treated larvae, which causes a substantial  
179 inhibition of epithelial ROS generation at the site of infection, as reflected by the decreased CellROX signal  
180 (**Figures 3A-B**). Noteworthy, this reduction of ROS production coincides with a reduced number of  
181 neutrophils mobilised towards bacilli in fish exposed to Roscovitine (**Figure 3A**). Additionally, confocal  
182 examination of Mabs-granuloma, a protective structure improving the control of Mabs infection<sup>48</sup>, revealed an  
183 abnormal granuloma architecture in Roscovitine-treated larvae, typified by reduced neutrophil infiltration  
184 (**Figure 3C**).

185 To further support zebrafish experiments, the neutrophil influx and activity were also evaluated in mice  
186 infected with Mabs then treated with Roscovitine or DMSO. Neutrophil numbers in lung compartments were  
187 enumerated at 6 days post-infection (dpi). As shown in **Figure 3D**, Roscovitine-treated mice exhibited  
188 reduced Ly6C<sup>hi</sup> / Ly6G<sup>hi</sup> staining, indicating that activated neutrophil amounts has decreased in lung after  
189 Roscovitine administration. Reduced relative numbers of activated neutrophil following Roscovitine treatment  
190 was confirmed by comparative analysis of cell composition in lung in these mice (**Figures 3E-G**). Of note, no  
191 changes in global neutrophil numbers were observed in zebrafish or mice, ensuring that the observed  
192 differences did not result from Roscovitine-induced neutropenia (data not shown).

193 Together these findings indicate that Roscovitine alters neutrophil mobilisation towards Mabs, likely by  
194 interfering with epithelial oxidative activity induced by Mabs infection, in addition to the critical role played in  
195 granuloma integrity with deleterious consequences such as extracellular mycobacterial multiplication<sup>48</sup>.

### 196 197 **Roscovitine exposure leads to exacerbation of Mabs infection *in vivo***

198 Neutrophils are dispensable for defence against Mabs infection<sup>48,49,52</sup>. The profound alteration of  
199 neutrophil chemotaxis to Mabs caused by Roscovitine, led us to hypothesize that Roscovitine may hamper  
200 host defence against Mabs and thus increase susceptibility to Mabs infection.

201 In order to test whether Roscovitine influences Mabs infection outcomes, intracellular Mabs killing was  
202 firstly investigated *ex vivo*, using primary macrophages obtained from both healthy and CF volunteers  
203 (**Figure 4A**). Relative luminescent units (RLU) analysis revealed a lower bacterial load in Mabs-infected  
204 macrophages treated with Roscovitine compared to vehicle alone at 24 hpi (**Figure 4B**), suggesting that  
205 Roscovitine can enhance macrophage Mabs killing in the context of CF. Interestingly, as previously reported,  
206 this might depend on the acidification of macrophages<sup>32</sup>, since Roscovitine improves acidification of  
207 macrophage lysosomes post Mabs infection, as shown by enhanced lysosomal fusion with intracellular Mabs  
208 and increased acidified lysosome numbers in macrophages (**Supp 3A-D**). To exclude direct Roscovitine-  
209 induced Mabs killing as the cause of enhanced mycobacterial clearance in macrophages, we evaluated  
210 minimum inhibitory concentrations. None of the Mabs variants showed direct Roscovitine susceptibility  
211 (**Table 1**), indicating that this compound has no direct antibacterial activity against Mabs. We demonstrate  
212 here that Roscovitine enhances macrophage-mediated intracellular killing of Mabs, likely by improving the  
213 lysosomal acidification in macrophages. However, little is known about the effect of Roscovitine on bacterial  
214 control *in vivo*.

215 Next, to establish whether Roscovitine treatment could affect the control of Mabs infection *in vivo*,  
216 zebrafish larvae were intravenously infected with Mabs<sup>50</sup> (**Figure 4A**). Our results indicated that both control-  
217 and *cflr*-MO exposed to Roscovitine displayed hyper-susceptibility to Mabs, correlating with increased larval  
218 mortality (**Figure 4C**) and higher bacterial loads (**Figure 4D**). Furthermore, microscopy observations showed  
219 that the increase in bacterial loads in Roscovitine-treated fish correlates with replicating extracellular  
220 bacteria, translating into increased number of abscesses and cord in the central nervous system of larvae<sup>49</sup>  
221 (**Figure 4D**). This is consistent with a reduced host defence and representative of severe Mabs infection in  
222 zebrafish<sup>48</sup>, and thus supports the hypothesis that Roscovitine treatment impedes the control of Mabs.  
223 Importantly, a similar impact of Roscovitine upon bacterial load was observed in mice infected with Mabs.  
224 Indeed, infected mice treated with Roscovitine displayed reduced ability to clear Mabs (**Figure 4E**) in the first  
225 days of infection, likely due to reduced neutrophil activity (**Figures 3D-F**). These phenotypes are in line with  
226 the increased bacterial loads in Roscovitine-treated mice infected with *Streptococcus pneumoniae*<sup>53</sup>.

227 Collectively, these results indicate that despite the favourable impact of Roscovitine on macrophage-  
228 mediated killing of Mabs, its activity increases *in vivo* susceptibility to Mabs infection, likely by hampering  
229 neutrophil chemotaxis towards infected sites and the nascent granuloma.

### 230 231 **DUOX2/NADPH-oxidase-driven neutrophil recruitment is crucial to control of Mabs *in vivo***

232 Release of H<sub>2</sub>O<sub>2</sub> gradients by epithelial cells through DUOX2/NADPH oxidase has been implicated in  
233 neutrophil chemotaxis to infected tissues<sup>51</sup>. Our results above suggest that epithelial ROS generation is  
234 required for neutrophil mobilization in response to Mabs infection (**Figure 3A**). We therefore investigated  
235 whether DUOX2 activity drives neutrophil recruitment to Mabs infection sites. DUOX2/NADPH oxidase was  
236 depleted<sup>54</sup> and the dynamic of neutrophils recruitment examined in *TgBAC(mpx:EGFP)i114* larvae.  
237 Inactivation of NADPH oxidase activity though injection of the *duox2* morpholino impaired neutrophil  
238 mobilization to the Mabs-infected somite (**Figures 5A-B**). This implies that DUOX2/NADPH oxidase-  
239 dependent ROS production is specifically required for early neutrophil chemotaxis towards Mabs.  
240 Additionally, confocal imaging underscored reduced number of neutrophil-associated granuloma in the  
241 absence of *duox2* signalling (**Figure 5C**). Importantly, loss of DUOX2 correlated with a defective neutrophil  
242 trafficking phenotype and abnormal granuloma architecture, similar to the one observed in infected fish  
243 treated with Roscovitine (**Figures 3B-C**). To characterise the role of *duox2* in Mabs infection control, both R  
244 and S variants were intravenously injected into control- and *duox2*-MO embryos. *duox2* knockdown resulted  
245 in a higher susceptibility to Mabs infections, associated with increased larval killing (**Figures 5D-G**) and  
246 enhanced bacterial loads, as demonstrated by determination of the fluorescent pixel count (FPC; **Figures**  
247 **5E-H**) and whole-larvae imaging (**Figures 5F-I**), further substantiating the importance of DUOX2/NADPH  
248 oxidase in controlling Mabs infection. Importantly, the increased susceptibility to Mabs infections in absence  
249 of DUOX2 activity correlates with enhanced extracellular bacterial multiplication, as evidenced by the higher  
250 number of abscesses (**Figures 5J-K**) as well as altered granuloma integrity (**Figures 5L-M**).

251 Together, these results indicate that release of DUOX2/NADPH oxidase-dependent ROS production at  
252 the infected sites represents a critical host defence against Mabs and demonstrate that the DUOX2 axis-  
253 dependent attraction of neutrophils is instrumental to efficiently contain bacteria within homeostatic  
254 granulomas, thereby preventing extracellular mycobacterial spread and limiting subsequent acute infection  
255 and larval mortality.

256

## 257 Discussion

258 Overactive neutrophil activity has been directly correlated with the onset of bronchiectasis and airway  
259 damage in CF, which in term causes lung function impairment and eventually death of people with CF. Thus,  
260 reducing the impact of neutrophil inflammation-mediated lung damage is a major concern in CF.

261 Among the attractive and innovative molecules to target pathways that are specific of the CF lung  
262 pathophysiology, Roscovitine shows multiple beneficial proprieties. In particular, Roscovitine stimulates  
263 macrophage bactericidal activity<sup>32</sup> and promotes neutrophil apoptosis<sup>11</sup> *ex vivo* in models of CF, suggesting  
264 that Roscovitine might simultaneously enhance bacterial killing and promote inflammation resolution,  
265 therefore prevent subsequent infectious and inflammatory lung damage in CF. However, evaluating  
266 Roscovitine in a CF animal model of infection or inflammation was awaited.

267 Here, moving from *ex vivo* through *in vivo* models of infection or inflammation, in both normal and CF  
268 conditions, we sought to determine the effect of Roscovitine on neutrophilic inflammation and how its activity  
269 influences the outcomes of infection and inflammation. Our findings indicate that Roscovitine exerts anti-  
270 inflammatory and pro-resolution effects in neutrophil response elicited by either Mabs infection or sterile  
271 injury. The proposed mechanism by which Roscovitine influences neutrophil trafficking suggests a reduced  
272 epithelial ROS burden due to its inhibiting property on DUOX2/NADPH-oxidase.

273 Whereas previous studies did not investigate Roscovitine effects early after induction of inflammation, our  
274 results reveal that Roscovitine especially attenuated neutrophil mobilisation rapidly after infection or injury.  
275 Importantly, our findings show that diminished neutrophil response coincided with a reduced epithelial  
276 oxidative activity in CF zebrafish treated with Roscovitine. This concurs with described reduced ROS  
277 production after Roscovitine treatment in a carrageenan-induced pleurisyin mouse model of inflammation<sup>55</sup>.  
278 Several mechanisms could be proposed to explain the action of Roscovitine on epithelial oxidative response,  
279 including a down-regulation of calcium release<sup>56</sup>, NF- $\kappa$ B<sup>26</sup> or TNF $\alpha$ <sup>55</sup> expression, as well as direct inhibition of  
280 DUOX2/NADPH-oxidase. Neutrophil mobilisation being predominantly elicited by DUOX2-mediated epithelial  
281 H<sub>2</sub>O<sub>2</sub><sup>36,57</sup>, our data suggest that by rebalancing epithelial ROS production, Roscovitine could be able to  
282 regulate early neutrophil mobilisation towards infected or inflamed tissue in CF.

283 Neutrophil apoptosis is impaired in CF<sup>7,58</sup> and can be reversed by Roscovitine in CF patient-derived  
284 neutrophils<sup>11</sup>. Furthermore, here we show that Roscovitine is able to induce *in vivo* apoptosis in CF zebrafish  
285 neutrophils. This study represents the first demonstration of the pro-apoptotic action of Roscovitine on  
286 neutrophils in an *in vivo* model of inflammation in the context of CFTR deficiency. CF-related inflammation is  
287 also determined by alterations in neutrophil reverse migration *in vivo*<sup>7</sup>. Reverse migration of neutrophil plays  
288 a crucial role in the resolution of inflammation in CF, since restoring this process using TIIA significantly  
289 rebalance neutrophil response in CFTR-depleted zebrafish<sup>7</sup>. Here we show for the first time, that Roscovitine  
290 can acts on CF zebrafish to restore the reverse migration ability of neutrophils, uncovering a new potential  
291 therapeutic mechanism for Roscovitine to drive inflammation resolution in CF. The mechanisms by which  
292 Roscovitine influences neutrophil reverse migration is particularly intriguing and deserve further attention.

293 CF zebrafish show impaired tissue regeneration after tail-fin amputation<sup>7</sup>, in part due to an unresolved  
294 neutrophilic inflammation, and which can be restored by pharmacological manipulation of neutrophil  
295 responses using TIIA<sup>7</sup>. Interestingly, while Roscovitine profoundly alleviates neutrophilic inflammation, our  
296 experiments show that Roscovitine does not improve tissue repair in injured fish. Possible explanations for

297 this finding include the following: (i) Roscovitine inhibits proteins CDK<sup>20</sup> and p38MAKP<sup>59</sup>, as well as epithelial  
298 ROS production : all these pathways are pivotal in the activation of regenerative processes; (ii) blocking  
299 CDK9 using Roscovitine delayed macrophage recruitment to injury<sup>60</sup>, an important cell population in the  
300 processes of tissue repair<sup>61</sup>; (iii) in contrast to TIIA, Roscovitine preferentially directs the neutrophil towards  
301 apoptosis rather than reverse migration. Following Roscovitine treatment, the large amount of apoptotic  
302 neutrophils generated could interfere with the efferocytosis potential of macrophages and thus might exert a  
303 prolonged local pro-inflammatory state delaying tissue repair.

304 With the slow development of new treatments and since Roscovitine is readily available and well-tolerated<sup>62</sup>,  
305 these findings could have significant therapeutic implications for potently targeting inflammation in CF lung  
306 disease, and thus may support currently therapeutic strategies or could be an alternative to existing anti-  
307 inflammatory approaches. These data also suggest Roscovitine might have beneficial effects on the pancreas  
308 destruction and CF-related diabetes<sup>63</sup> or gastrointestinal and colorectal cancers in CF<sup>64,65</sup>. While CF is  
309 principally characterised by pulmonary infection and inflammation, intestinal disruption involving chronic  
310 inflammation is also a frequent feature<sup>64</sup>. In CF, epithelial surfaces produce an increased ROS burden<sup>7</sup> with  
311 potential genotoxic consequences. While ROS are directly mutagenic to DNA, H<sub>2</sub>O<sub>2</sub> produced in epithelia is  
312 a potent chemoattractant source for neutrophils, driving local inflammation<sup>36</sup>, itself a known driver of  
313 tumourigenesis<sup>66</sup>. Moreover, ROS production is also a proliferative signal in many epithelial cell types<sup>67</sup>.  
314 Interestingly, Duox2 knockout significantly alleviate intestinal inflammation in a mouse model of ileocolitis<sup>68</sup>,  
315 suggesting that targeting DUOX2-mediated ROS production might show promise in the treatment of  
316 gastrointestinal cancer in people with CF. Firstly known for its anti-cancer properties, Roscovitine is currently  
317 being tested in several phase I and II clinical trials against human cancers<sup>69</sup>. So, by restoring normal level of  
318 inflammation in CF, Roscovitine might also, by reducing cell proliferation, epithelial ROS-mediated  
319 mutagenesis and inflammation, prevent cancer in CF patients.

320 Mabs infections are associated with severe pneumonia and accelerated inflammatory lung damage in CF  
321 patients<sup>70,71</sup>. In line with results previously obtained<sup>31,32</sup>, Roscovitine reduces intracellular bacterial loads in  
322 both WT and CF macrophages infected with Mabs, likely by enhancing their ability to kill bacteria. As  
323 intracellular bacterial destruction by professional phagocytes is crucial to control Mabs infection<sup>6,72</sup>, perhaps  
324 stimulating antibacterial activity using Roscovitine and thereby precluding the establishment of an acute  
325 infection could be a therapeutic strategy in CF-related Mabs infection. Roscovitine stimulates macrophage  
326 bactericidal activity by restoring intra-phagolysosome acidic pH<sup>31,32</sup> (which is abnormally high in CF  
327 macrophages<sup>73</sup>). Having shown that professional phagocytes acidify phagosomes to efficiently control  
328 Mabs<sup>72,74,75</sup>, Roscovitine-mediated intra-phagosomal acidification could account for the Mabs infection  
329 phenotype. Interestingly, Roscovitine was found to inhibit Nox2-mediated ROS production in nociceptive  
330 neurons through the blockade of Cdk5<sup>55</sup>. Nox2-mediated ROS production in macrophages and neutrophils is  
331 another important antibacterial actor against Mabs<sup>6</sup>. These results could suggest that phagosomal  
332 acidification is a more potent microbicidal mechanism against Mabs than ROS activity in phagocytes. At this  
333 stage, the differential importance of acidic and oxidative defences in the control of Mabs remains to be firmly  
334 established. It will be interesting to see whether Roscovitine influences oxidative responses against Mabs.  
335 Answering these questions will provide evidence on the most interesting antibacterial mechanisms that could

336 be enhanced therapeutically to better deal with Mabs infections. In addition, whether Roscovitine influences  
337 the antibacterial defence of neutrophils has not yet been tested and remains to be addressed.

338 Unexpectedly, while Roscovitine was able to enhance Mabs killing *ex vivo*, a substantial exacerbation of  
339 Mabs infection was found in mice and zebrafish treated with Roscovitine. In particular, Mabs-infected  
340 zebrafish rapidly succumbed when exposed to Roscovitine in both WT and CF conditions. Hyper-  
341 susceptibility to Mabs due to the Roscovitine exposure is associated with increased extracellular Mabs  
342 multiplication and abnormal granuloma maintenance which are representative of a profound impairment in  
343 Mabs control<sup>48,49</sup>. Importantly, this increased susceptibility to Mabs coincides with reduced neutrophil  
344 mobilization and activity towards infected compartments in mouse and zebrafish. Our previous work  
345 highlighted the critical role of neutrophils in the control of Mabs infection by phagocytosing and killing  
346 bacilli<sup>47,76</sup> and by favouring the formation of granulomas able to restrict extracellular multiplication of Mabs<sup>77</sup>.  
347 Zebrafish failed to mount a normal epithelial oxidative response to pathogens when treated with Roscovitine,  
348 strongly suggesting that Roscovitine affects ROS-driven chemotaxis guiding neutrophils to the nascent  
349 granulomas, potentially promoting extracellular Mabs growth and thereby an acute infection.

350 Although studies postulated that infection-associated neutrophil recruitment is dispensable to epithelial  
351 ROS production<sup>78</sup>, we demonstrate the capacity of neutrophils to migrate in DUOX2-derived ROS dependant  
352 manner in response to Mabs, that would be directly involved in the formation of protective granulomas. This  
353 result shows for the first time that host-derived epithelial ROS signalling, mediated by DUOX2/NADPH  
354 oxidase, can prime neutrophil chemotaxis to Mabs infection and therefore defines a critical role for DUOX2  
355 activity in the control of Mabs infection. As a consequence, oxidative activity blockade by Roscovitine  
356 increases the risk of impeding host innate immune response and therefore promote an overwhelming Mabs  
357 infection. However, since Roscovitine showed enhanced efficacy in combination with other existing  
358 therapeutics such as CFTR modulators<sup>31</sup>, Roscovitine will likely diminish the severity of inflammatory lung  
359 injury driven by microbial components, host inflammatory mediators as well as genetic defect in CFTR, and  
360 accelerated recovery in the context of antibiotic therapy in CF patients.

361 In addition, while apoptosis is essential for neutrophil shutdown and initiating inflammation resolution, the  
362 reduced number of neutrophils due to the pro-apoptotic Roscovitine action may also affects the ability of  
363 immune system to efficiently respond to Mabs infection. In contrast, reverse-migrated neutrophils were found  
364 able to mount a response to *S. aureus* infection *in vivo*<sup>79</sup>. At this stage, the role of neutrophil reverse  
365 migration in the process of infection and inflammation in CF remains to be fully characterised. Reverse  
366 migration could have the potential to be deleterious, allowing localised infection or inflammation to  
367 disseminate<sup>80</sup>. Alternatively, encouraging neutrophil egress from infected or inflamed sites could serve as a  
368 pro-resolving mechanism<sup>7,37</sup>. Answering these questions in CF pulmonary disease will determine how best to  
369 harness apoptosis or reverse migration for therapeutic purposes to drive inflammation resolution while  
370 minimizing the risk of impaired innate immunity in people with CF.

371 To conclude, CFTR mutations affect mucus properties, inflammatory processes and antibacterial  
372 defences. These different aspects are intertwined: treating one of these features has consequences on the  
373 other two. Given its anti-oxidative action, the application of Roscovitine in CF could induce counterproductive  
374 and needs therefore to be further studied.

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396

397 **Authorship Contributions**

398 AB conceived the study and wrote the manuscript with input from SAR, RAF and J-LH. RAF, J-LH and AB  
399 designed experiments and analysed data. RAF, J-LH and AB guided and supervised the work. CL provided  
400 zebrafish tools. VLM, DR-R, SG, C-MD, AAAS and AB performed experiments. All authors contributed to the  
401 article and approved the submitted version.

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403

404 **Disclosure of Conflicts of Interest**

405 The authors declare that the research was conducted in the absence of any commercial or financial  
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## 586 Figure legends

587

### 588 **Figure 1. Roscovitine-reduced epithelial oxidative activity rebalances early neutrophil mobilisation at** 589 **wound in CF zebrafish model**

590 (A-B) WT, *cfr*<sup>-/-</sup> and *cfr* MO *TgBAC(mpx:EGFP)*i*114* larvae were pre-treated with of Roscovitine, DPI (as  
591 positive control) TIIA (as negative control) or DMSO (as mock control) prior to tail fin amputation procedure,  
592 then injured and immediately put back in treatments for 4 h. Neutrophil number at the wound (dotted lines)  
593 was observed and enumerated at 4 hpA under a fluorescence microscope. (A) Neutrophil recruitment assay  
594 (*n*= 21, Two-Way ANOVA with Dunnett's post-test, error bars represent SEM). (B) Representative number of  
595 neutrophils at wound in Roscovitine- versus DMSO-treated *cfr* MO zebrafish (Scale bars, 200 μm). (C-D) *cfr*  
596 MO stained with CellROX<sup>®</sup> to label H<sub>2</sub>O<sub>2</sub> generation. Means ± SEM ROS intensity (C) and associated  
597 pseudocolored photomicrographs (D) of injured tails revealing oxidative activity at 30 min post-amputation  
598 (mpA) in *cfr* MO treated with Roscovitine (*n* = 12, Mann Whitney test; Scale bars, 200 μm).  
599

600

### 601 **Figure 2. Roscovitine accelerates inflammation resolution *in vivo* both by inducing neutrophil** 602 **apoptosis and reverse migration**

603 (A-B) Control-Mo or *cfr*-MO *TgBAC(mpx:EGFP)*i*114* were injured and treated from 4 hpA with Roscovitine or  
604 of TIIA. (A) Neutrophil number at the wound was observed and counted at 8 hpA (*n*=21, Two-Way ANOVA  
605 with Tukey's multiples comparison test). (B) Representative number of neutrophils remaining at wounds  
606 (Scale bars, 200 μm). (C-D) injured-*cfr* MO larvae were treated with Roscovitine from 4 hpA and stained  
607 with TUNEL/TSA to label apoptotic cells (C) Neutrophil apoptosis quantification at 8 hpA (*n*= 15, Fisher *t*-  
608 test). (D) Representative confocal pictures of injured tails (Scale bars, 50 μm) revealing the proportion of  
609 apoptotic neutrophils at the wound at 8 hpA. (E-F) Reverse-migration in *cfr* MO  
610 *Tg(mpx:gal4)sh267;Tg(UASkaede)*i*222* after Roscovitine treatment. At 4 hpA, neutrophils at site of injury  
611 were photoconverted then the numbers of photoconverted cells (red) that migrate away (white dotted box)  
612 from the photoconverted area (blue dotted box) were time-lapse imaged and quantified over 4 hours by  
613 confocal microscopy (E). (F) Plot showing the number of photoconverted neutrophils leaving the wound over  
614 4 hours post photoconversion (hpc). Line of best fit shown is calculated by linear regression. *P*-value shown  
615 is for the difference between the 2 slopes (*n*= 12, performed as 3 independent experiments). (G)  
616 Representative confocal imaging of injured tails showing the kinetics of photoconverted neutrophils that

616 move away from the area of injury over inflammation resolution.

617

618 **Figure 3. Roscovitine impedes neutrophil trafficking during Mabs infection**

619 (A-B) WT *Tg(mpx:eGFP)i114* larvae were treated with Roscovitine or DMSO then infected into the somite  
620 with  $\approx 100$  Mabs R expressing dtTomato. Infected larvae are stained with CellROX<sup>®</sup> to label ROS production.

621 Representative epithelial oxidative response (arrow) and number of neutrophils at infection site in  
622 Roscovitine- versus DMSO-treated larvae at 3 hours post-infection (hpi) (Scale bars, 75  $\mu\text{m}$ ). (B) Means  $\pm$   
623 SEM ROS intensity at the site of infection (2 hpi,  $n = 8$ , student t test). (C) Confocal images showing the  
624 representative repartition of neutrophil-associated Mabs granuloma in larvae treated with Roscovitine  
625 compared with DMSO-exposed animals (Scale bars, 10  $\mu\text{m}$ ).

626 (D-G) Mice were intravenously infected with R Mabs then treated with 50  $\mu\text{M}$  Roscovitine or DMSO at 1dpi.  
627 At 6 dpi neutrophils are isolated from the lung of mice and analysed by flow cytometry. (D) Representative  
628 dot-plots showing the expression of Ly6C<sup>hi</sup> / Ly6G<sup>hi</sup> (activated neutrophil) among neutrophils. Graphs showing  
629 the mean  $\pm$  SEM absolute (E) and relative (F) number of activated neutrophils, and related ratio of activated  
630 neutrophils (G) in lungs ( $n=5$ , unpaired Student's *t* test, representative of 3 independent experiments).

631

632 **Figure 4. Roscovitine exacerbates Mabs infection in zebrafish and mouse model of infection**

633 (A) The effect of Roscovitine on Mabs infection outcomes was evaluated in primary human macrophage (B),  
634 zebrafish (C-D) and mouse (C) model of infection. (B) Monocyte-derived primary human macrophages were  
635 infected at a MOI 1:1 with bioluminescent Mabs (Mabs-lux) for 2 hours. Extracellular bacteria were washed  
636 off and fresh media containing Roscovitine or DMSO added. At each specified time-point, cells were lysed  
637 and viable intracellular bacteria quantified as relative luminescent units (RLU). Roscovitine enhances  
638 intracellular Mabs killing in macrophages obtained from both healthy volunteers and CF patients (One-Way  
639 ANOVA with Dunnett's post-test). (C-D) Control MO or *cftr* MO were intravenously infected with  $\approx 100$ -150  
640 Mabs R expressing *tdTomato*. From one day post-infection (dpi) larvae were treated with Roscovitine or  
641 DMSO. (C) Survival analysis of Control MO (left) or *cftr* MO zebrafish (right). Data are plotted as percentage  
642 of surviving animals over a 10 days period ( $n=30$ , Mantel-Cox Log-rank test, average of two independent  
643 experiments). (D) Representative whole-larvae imaging of Control MO (left) or *cftr* MO zebrafish (right) at 3  
644 dpi (Scale bars, 200  $\mu\text{m}$ ). (E) Mice were intravenously infected with Mabs then treated 24 hours later with 10  
645 and 50  $\mu\text{M}$  Roscovitine or DMSO. The surviving bacteria were enumerated after 6 dpi by CFU analysis.  
646 Results are expressed as  $\log_{10}$  units of CFU per organ at 1 (before treatment administration) and 6 dpi (Two-  
647 Way ANOVA with Dunnett's post-test).

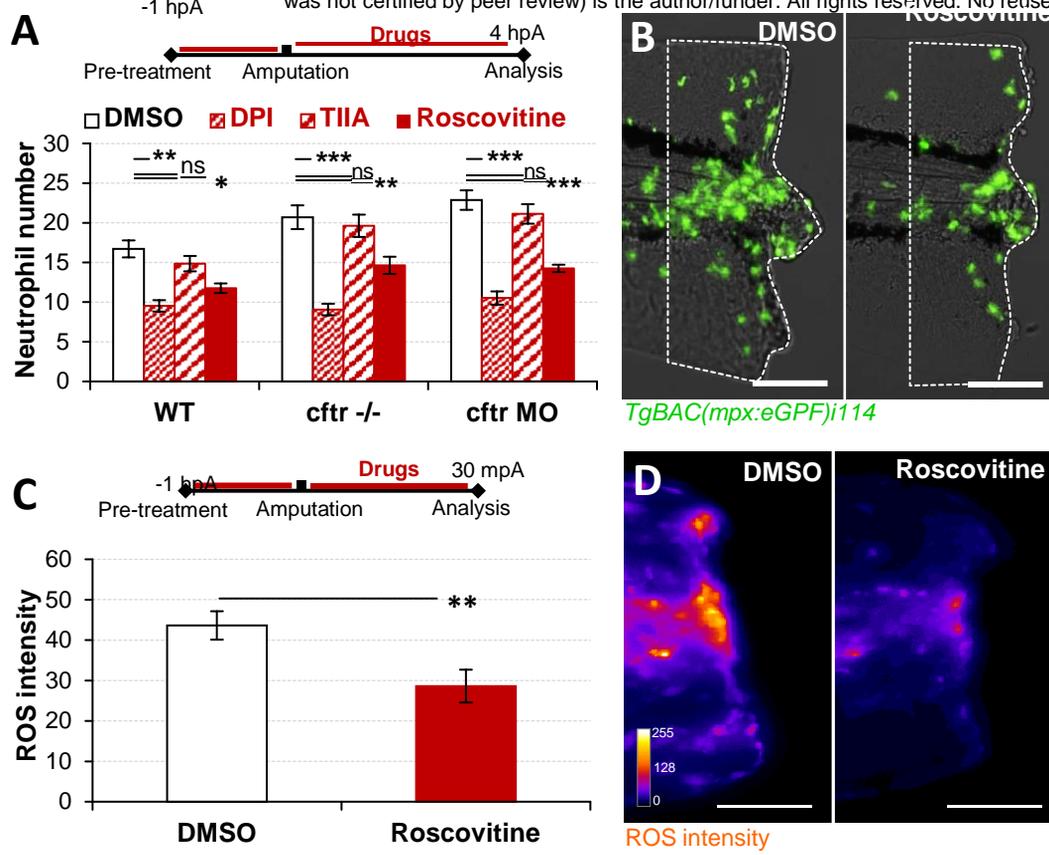
648

649 **Figure 5. Epithelial oxidative response-dependent recruited neutrophils restricts Mabs infection**

650 (A-B) Control MO or *duox2* MO *Tg(mpx:eGFP)i114* larvae were infected into the somite with  $\approx 100$  CFU Mabs  
651 S expressing *dtTomato*. (A) Mean  $\pm$  SEM number of neutrophils mobilized to the infection site at 3 hpi ( $n=$   
652 20, average of two independent experiments) and (B) representative neutrophil-associated site of infection  
653 (Scale bars, 75  $\mu\text{m}$ ). (C-M) Control MO or *duox2* MO were intravenously infected with  $\approx 100$ -150 CFU of  
654 Mabs R or S expressing *tdTomato*. (C) Confocal images showing the representative repartition of neutrophil-  
655 associated *Mabs* granuloma in Control MO versus *duox2* MO (Scale bars, 10  $\mu\text{m}$ ). (D and G) Survival

656 analysis of R- (D) or S-infected larvae (G). Data are plotted as percentage of surviving animals over a 10  
657 days period (n=60, Mantel-Cox Log-rank test, Average of three independent experiments). (E and H) Mean  
658 fluorescent pixel counts (FPC) of 3 dpi larvae infected by either R (E) or S (H) variants. Results are  
659 expressed as log<sub>10</sub> units of FPC per fish. (F and I) Representative images of R- (F) or S-infected larvae (I) at  
660 3 dpi (Scale bars, 200 μm). (J and K) Percentage of 3 dpi infected larvae with abscess (J) from three  
661 independent experiments (n=30) and associated mean ± SEM number of abscess per infected animal (K).  
662 (L-M) Kinetic of granuloma formation in whole embryos over a 4-day infection period (L) from three  
663 independent experiments (n=30) and associated mean ± SEM number of granuloma per infected animal (M).  
664 Statistical significance: Mantel-Cox Log-rank test (D and G), two-tailed unpaired Student's t test (B, E, H and  
665 K), Fisher's exact test of a contingency table (J and L) or Two-Way ANOVA with Tukey's multiples  
666 comparison test (M).

667



**Fig1**

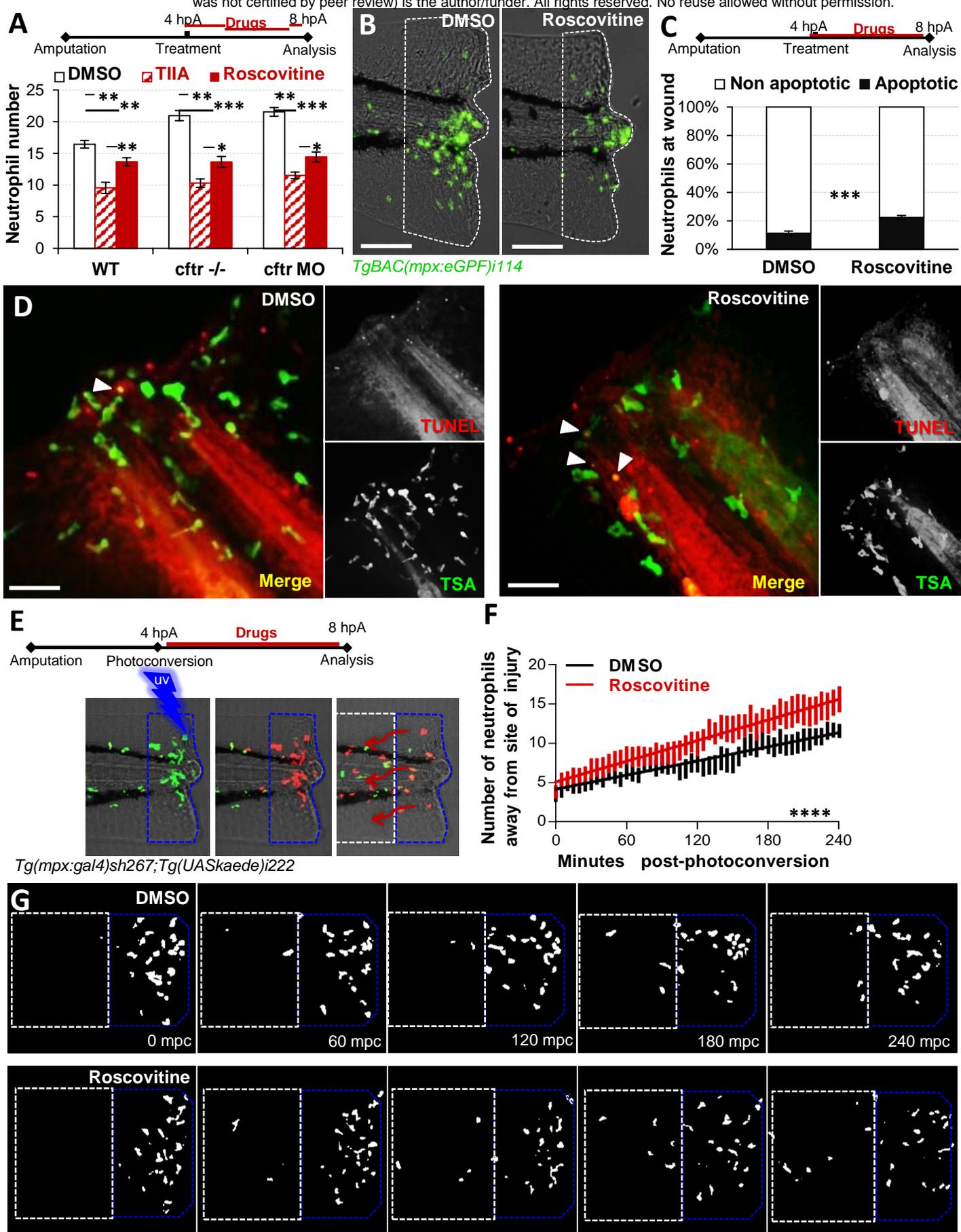
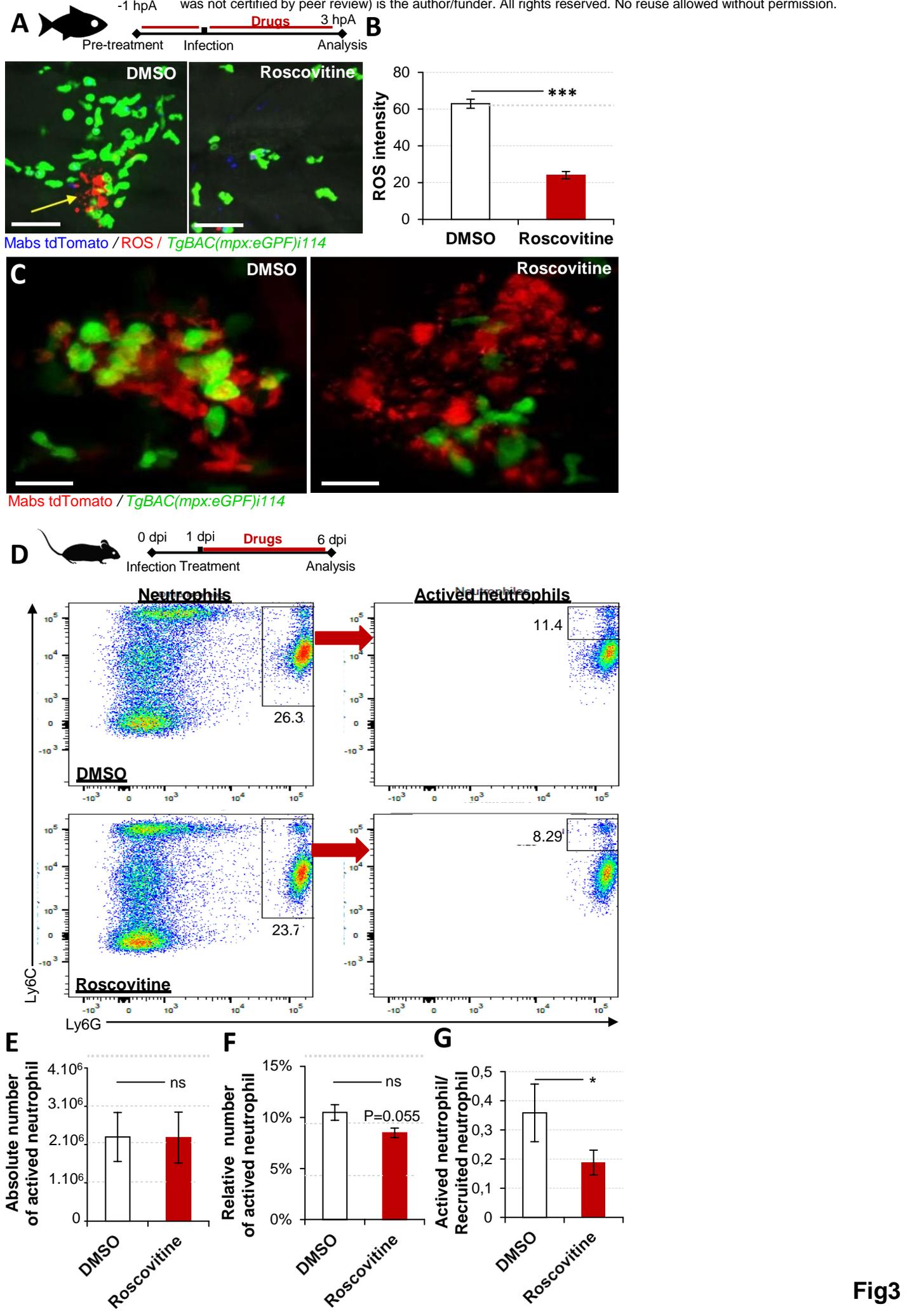


Fig2



**Fig3**

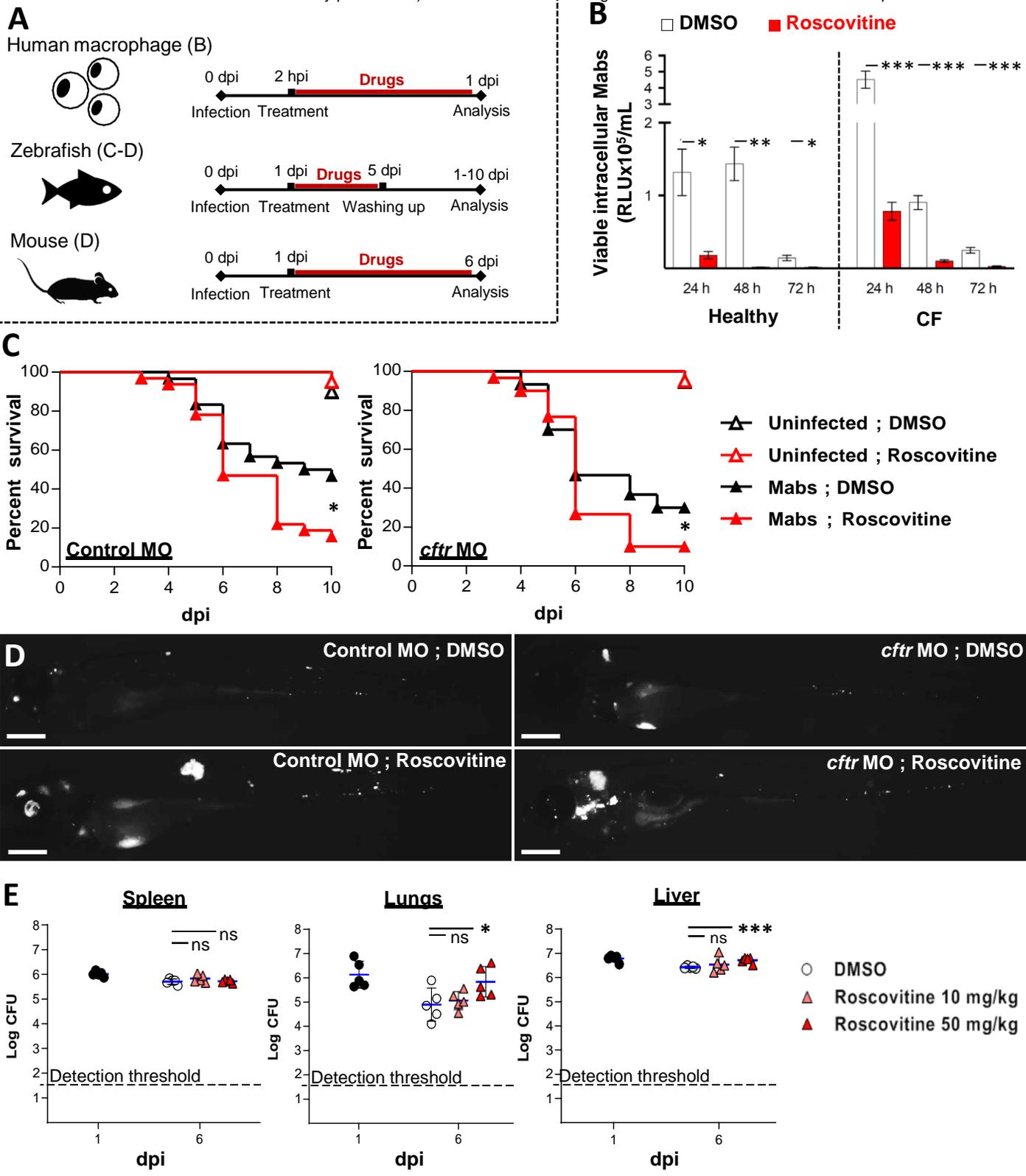
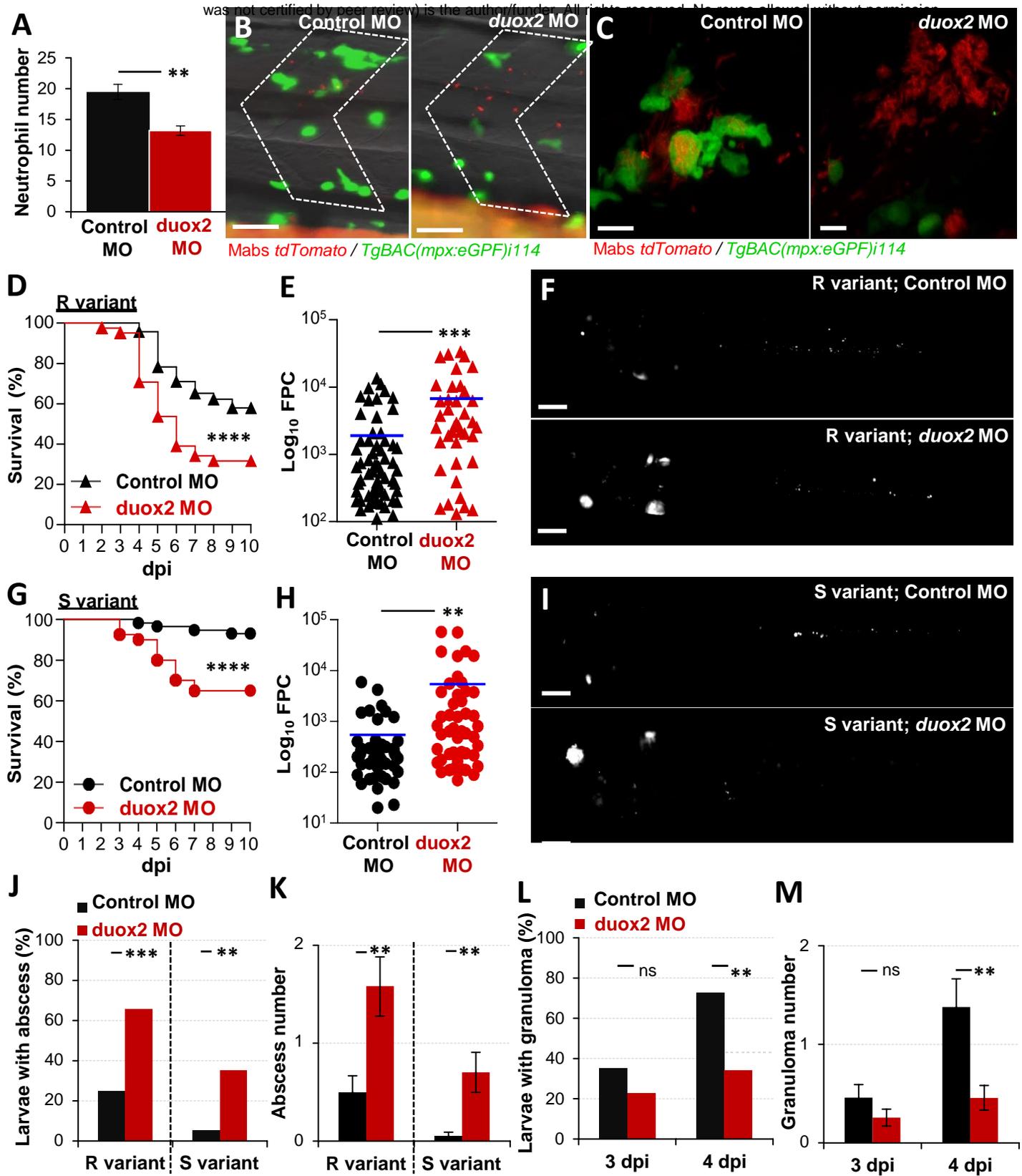


Fig4



**Fig5**